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14. ABSTRACT

New approaches are required to control multi-drug resistant (MDR) bacterial infections in military medical facilities, as injured Warfighters are highly susceptible to such infections. 2-Aminoimidazole compound that, at sub-micromolar levels and in conjunction with conventional antibiotics, will inhibit and disperse biofilms from both Gram-positive and Gram-negative pathogens, and re-sensitize MDR variants of these pathogens to FDA approved antibiotics are being developed and a number analogues displaying activity at low micromolar concentrations have been identified. The lead compound does not exhibit eukaryotic toxicity, both alone and in combination with antibiotics, at concentrations well above those at which it exhibits anti-biofilm and antibiotic resistance suppression activity. This now allows us to take the lead compounds identified from the in vitro screening assays into in vivo experiments using animal models of infection (mouse puncture and pig partial thickness and rat osteomyelitis) developed at WRAIR.

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Multi-drug resistance, biofilm, antibiotic resistance suppression, 2-amnoimidazole

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INTRODUCTION:

New approaches are required to control multi-drug resistant (MDR) bacterial infections in military medical facilities, as injured Warfighters are highly susceptible to such infections. MDR bacterial infections can cause sepsis, cellulitis and skin abscesses, pneumonia, toxic shock syndrome, osteomyelitis, and endocarditis among other symptoms. Serious cases result in organ failure (especially kidney), loss of limbs (via amputation) and death.

Presenting a tremendous impediment for the treatment of MDR infections are bacterial defense mechanisms such as biofilm formation and antibiotic resistance. Bacteria within a biofilm are upwards of 1000-fold more resistant to antibiotics than their planktonic (free-floating) counterparts and they are inherently insensitive to the host immune response. Antibiotic treatment is further compromised by the acquisition of antibiotic resistance genes such as ß-lactamases, multidrug efflux pumps, and antibiotic modifying enzymes.

The purpose of this research is to simultaneously address both multi-drug resistance and biofilm development. The overall goal of this research is therefore to identify a 2-AIT conjugate that, at sub-micromolar levels and in conjunction with conventional antibiotics, will: 1) disperse biofilms from both Gram-positive and Gram-negative pathogens, focusing on *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* (MRSA), and MDR *Acinetobacter baumannii* (MDRAB); 2) re-sensitize MDR variants of these pathogens to the effects of at least one conventional, FDA-approved antibiotic; and 3) exhibit acceptable toxicological properties for use on the Wounded Warrior. Based upon this goal, the Aims of this research proposal are to:

1. Develop highly active 2-AIT derivatives based upon a current lead compound.
2. Evaluate 2-AIT derivatives for their ability to inhibit and disperse bacterial biofilms, as well as suppress antibiotic resistance.
3. Evaluate active 2-AIT derivatives for in vitro toxicity. Models will include epidermal cell toxicity, model organism toxicity (*Caenorhabditis elegans*), and hemolysis.
4. Evaluate active 2-AIT derivatives for in vivo activity in collaboration with COL. Craft (Director, Wound Infections/Diagnostics) and his team at Walter Reed Army Institute of Research (WRAIR). We will employ the use of three separate models, mouse puncture and pig partial thickness wound models that will evaluate the topical efficacy of the 2-AIT derivatives. We will also evaluate the 2-AIT derivatives in a rat osteomyelitis model of infection.

RESEARCH ACCOMPLISHMENTS

Aim 1. Develop highly active 2-AIT derivatives based upon a current lead compound.

Library Generation:
A number of distinct libraries of novel analogues of the initial lead compound **2-AIT-1** and a second lead compound **2-AIT-2** (Figure 1) subsequently identified from screening of our in-house library for suppression of MRSA resistance to penicillin G have been designed and synthesized. Synthetic routes are summarized below, or in the referenced literature. Structures of all analogues synthesized are detailed below.

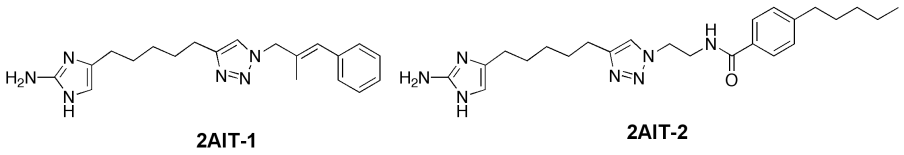


Figure 1. Lead compounds **2AIT-1** and **2AIT-2**

Library 1. A library of 14 analogues of **2-AIT-1** based upon structural modification of the aryl appendage, has been synthesized through the use of Suzuki–Miyaura coupling[1] (Figure 2).

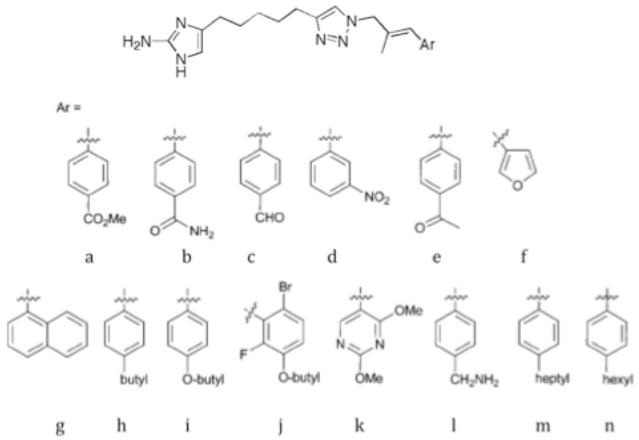


Figure 2. Library 1, **2-AIT 1** analogues with varied aryl appendages.

Library 2. A second library of 22 analogues of the initial lead compound **2-AIT-1**, in which diversity was introduced at the 5-position on the 2-AI head group, was synthesized, along with corresponding analogues of compound **g** from Library 1 (Figure 3). This synthesis exploited the addition of Grignard reagents to a Weinreb

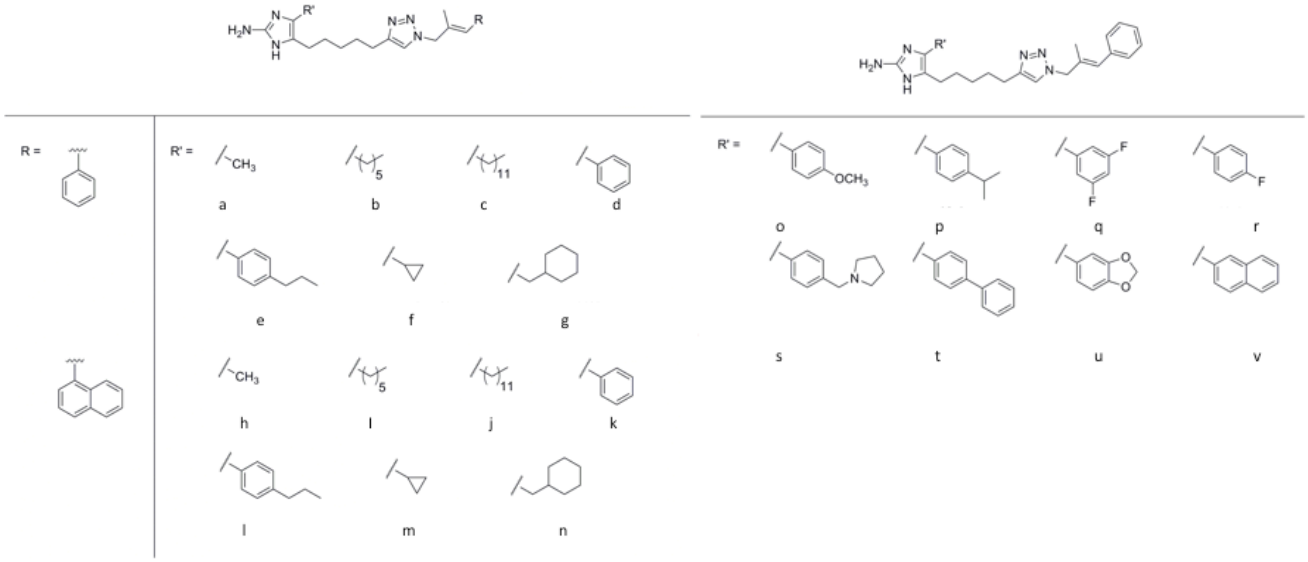


Figure 3. Library 2, 4.5-disubstituted **2-AIT 1** analogues.

amide to introduce diversity at the 5-position of the 2-AI.[2]

Library 3. As an alternative approach to generate diversity on the 2-AI scaffold, a third library has been synthesized, consisting of 16 triazole acyl derivatives[3] (Figure 4).

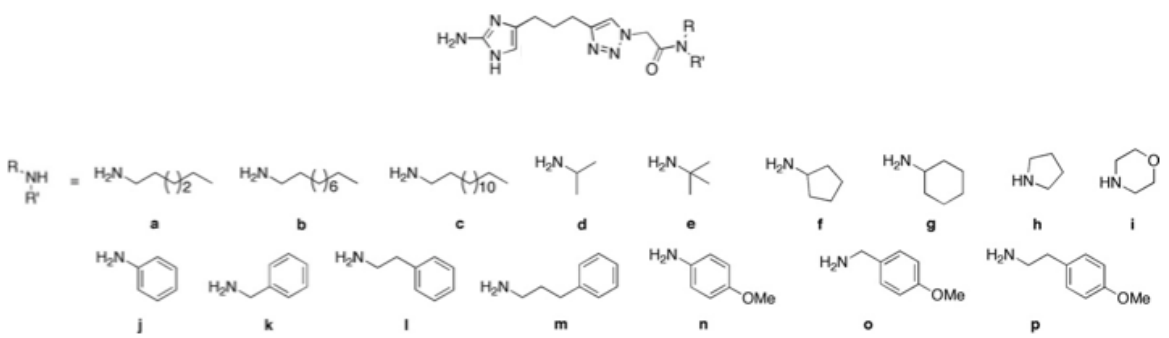


Figure 4. Library 3, azide acyl derivatives of **2-AIT-1**

Following the identification of a second lead structure **2-AIT-2** (Figure 1) with improved resistance suppression activity towards MRSA compared to our initial lead **2-AIT-1**, four libraries of analogues of **2-AIT-2** are being developed incorporating various substitution patterns about the 2-aminoimidazole ring (Figure 5).

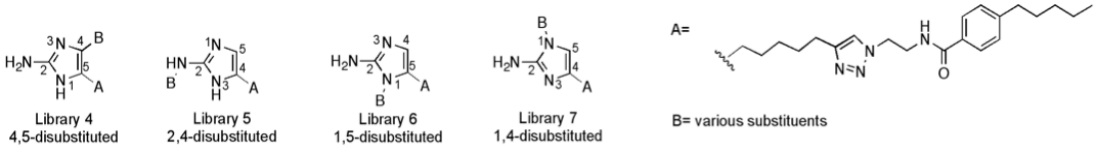


Figure 5. Design of analogues of the current lead compound **2-AIT-2**

Library 4. A library of 23 4,5-disubstituted analogues of **2-AIT-2** has been developed using chemistry developed in the synthesis of library 2 of **2-AITA-1** analogues described above (Figure 6).

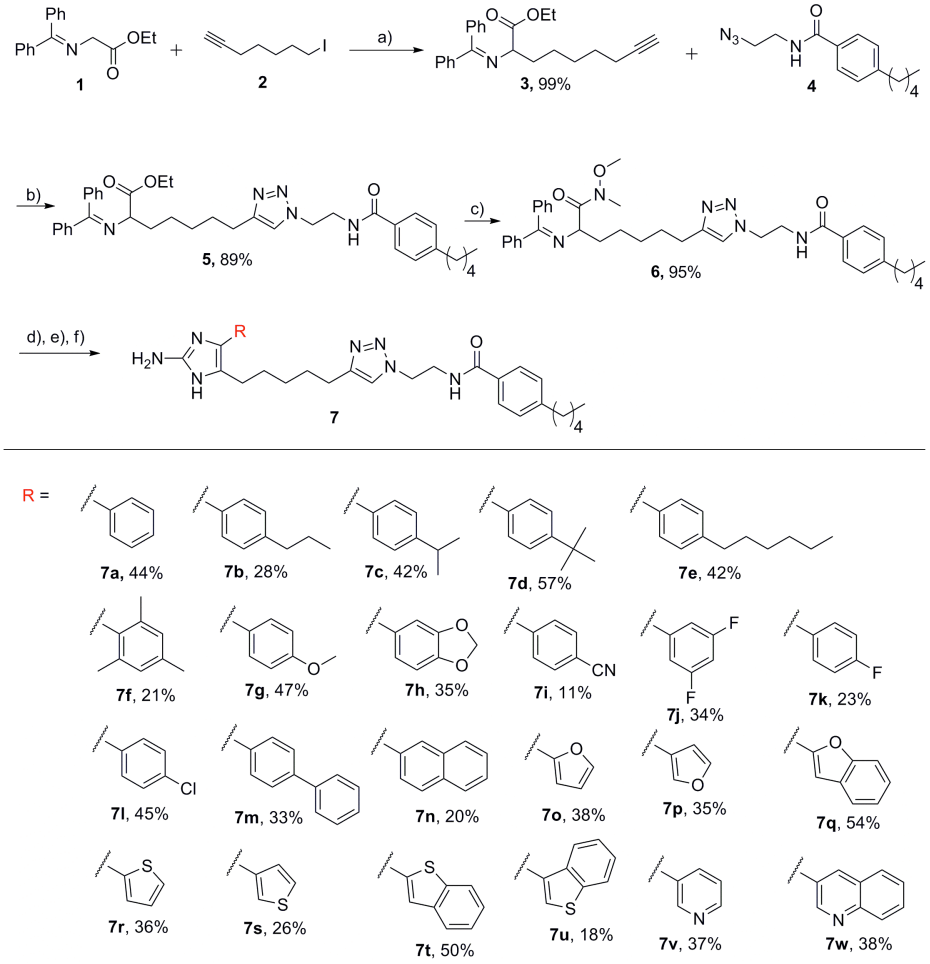


Figure 6. Library 4, 4,5-disubstituted **2-AIT 2** analogues.

Library 5. A library of 2,4-disubstituted analogues of **2-AIT-2**, in which diversity is introduced via monosubstitution of the exocyclic amino position of a Boc protected 2-aminoimidazole has been assembled, consisting of 16 analogues (Figure 7).

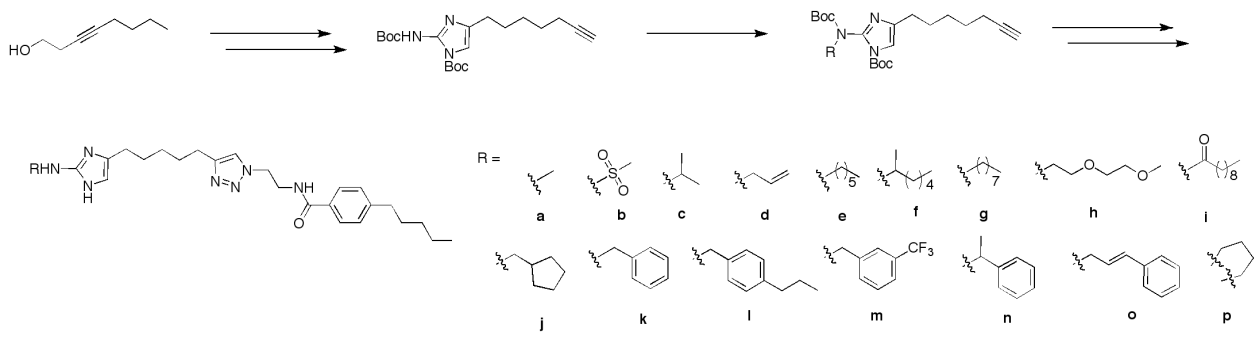


Figure 7. Library 5, 2,4-disubstituted **2-AIT 2** analogues.

Library 6. A library of 15 1,5-disubstituted analogues of **2-AITA-2** possessing various substituted benzyl groups at the 1-positon (Figure 8) has been constructed using chemistry developed in the synthesis of series of simple 1,5-substituted 2-aminoimidazoles.[4]

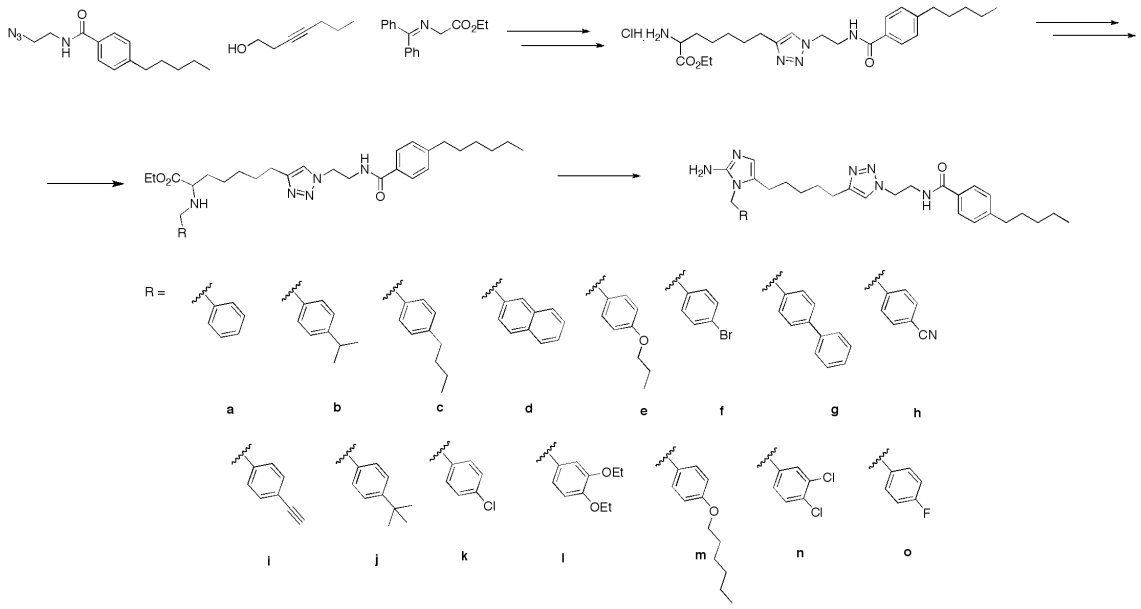
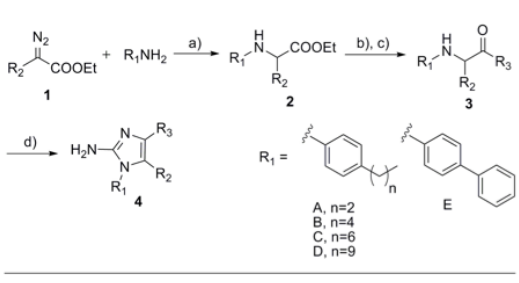


Figure 8. Library 6, 1,5-disubstituted **2-AIT 2** analogues.

Library 7. A synthetic route to access a library of 1,4-disubstituted analogues of **2-AITA-2** has been developed (Figure 9). Initial attempts to access analogues from a common epoxide intermediate (Figure x), by opening of the epoxide with primary amines, followed by oxidation of the resulting alcohol to a ketone, proved unsuccessful. An alternative route has therefore been designed and implemented which involves conversion of the carboxylic acid depicted in Figure 6 to an α-bromo ketone, followed by displacement of the bromide with a variety of primary amines. Cyclization of the subsequent amino ketones with cyanamide affords the 1,4-disubstituted 2-aminoimidazoles.

Library 8. A methodology for the rapid assembly of 1,4,5-trisubstituted 2-aminoimidazoles has been developed in order to expand our ability to access multiple substitution patterns about the 2-aminoimidazole ring.[6] Similar to the approaches to libraries 4 and 6 above, 1,4,5-substituted-2-aminoimidazoles were assembled from cyclization of *N*-substituted α-amino ketones with cyanamide. These α-amino ketones were readily prepared from N-H insertion between an appropriate diazoester and a commercially available amine, followed by conversion of the ester to the Weinreb amide and Grignard addition (Figure 7). Screening of conditions for the N-H insertion reaction revealed $[RuCl_2(p\text{-cymene})]_2$ in DCM to be the most effective. The resulting *N*-substituted α-amino esters were converted to the desired 1,4,5-substituted-2-aminoimidazoles using similar chemistry to that developed for the synthesis of library 4 analogues. The pilot library (library 8) is depicted in Figure 9. The modular nature of this approach allows for rapid assembly of diverse analogues for preliminary biological screening.



Entry	R_1	R_2	R_3	Entry	R_1	R_2	R_3
a	Ph	Ph	Me	i	D	Ph	Ph
b	Ph	Ph	*n*-Hexyl	j	E	Ph	Hexyl
c	Ph	Ph	Ph	k	E	Ph	Ph
d	C	Ph	Me	l	Ph	*p*-Tol	Hexyl
e	C	Ph	*n*-Hexyl	m	Ph	*p*-Tol	Ph
f	C	Ph	Ph	n	A	Ph	Me
g	D	Ph	Me	o	B	Ph	Me
h	D	Ph	*n*-Hexyl				

Figure 9. Synthesis and pilot library of 1,4,5-trisubstituted 2-aminoimidazoles

<u>Aim 2. Evaluate 2-AIT derivatives for their ability to inhibit and disperse bacterial biofilms, as well as suppress antibiotic resistance.</u>

Biofilm inhibition and dispersion and resistance suppression data for the compounds described above are tabulated in the supporting data section. The activity of the most efficacious compounds will be summarized in this section.

2-AIT-2 activity.

A second lead compound **2-AIT-2** was identified from screening of our in-house library for suppression of MRSA resistance to penicillin G. This compound exhibits biofilm inhibition and dispersion activity against MRSA and MDRAB as well as a number of other bacterial strains.[5] MRSA (ATCC BAA-44) IC_{50} (concentration at which the compound inhibits 50% of the biofilm compared to an untreated control)= 4.3 μM, EC_{50} (concentration at which the compound disperses 50% of the biofilm compared to an untreated control)= 80.6 μM, MDRAB (ATCC BAA-1605) IC_{50} = 15.1 μM, EC_{50} = 17.2 μM.

Similarly to **2-AIT-1**, **2AIT-2** reduced the MIC of penicillin G against MRSA (ATCC BAA-44) by eight-fold (from 32 μg/mL to 4 μg/mL). **2AIT-2** exhibits increased suppression of MRSA resistance to oxacillin compared to **2AIT-1**, reducing the MIC by eight-fold (from 32 μg/mL to 4 μg/mL) while **2AIT-1** reduced the oxacillin MIC against this MRSA strain by two-fold (from 32 μg/mL to 16 μg/mL). The oxacillin re-sensitization activity of this compound has been further characterized through the construction of time-kill curves (Figure 10).

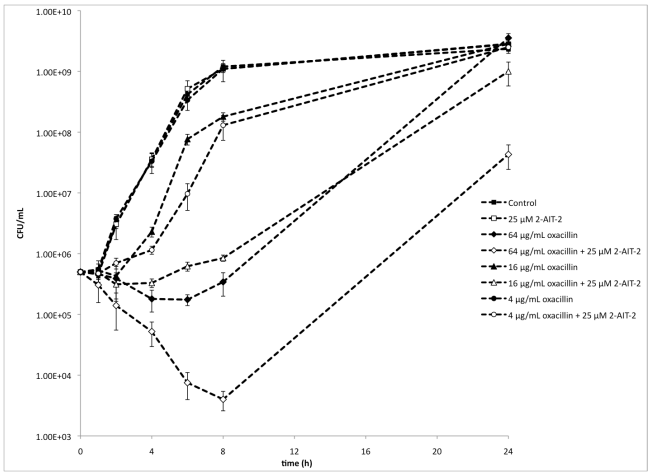


Figure 10. Time kill curves for MRSA (ATCC BAA-44) with oxacillin in the presence and absence of **2-AIT-2**

Time kill curves allow quantitative measurement of the magnitude of bacterial growth reduction as a function of time. To construct the time kill curves MRSA cultures were grown in various concentrations of oxacillin and **2-AIT-2**, bacteria were plated at set time points and the number of viable CFUs was enumerated. Addition of **2-AIT-2** dramatically reduces the number of CFU compared to treatment of the bacteria with oxacillin alone, particularly at the 8 h time point, by which time for oxacillin alone the number of CFUs had begun to increase. Oxacillin, at 4 μg/mL in the presence of **2-AIT-2**, reduced the number of CFUs by a greater amount than oxacillin alone did at 16 μg/mL.

2-AIT-2 suppressed MRSA resistance to all ß-lactam antibiotics studied with the exception of the monobactam aztreonam, which does not possess activity against Gram-positive bacteria. **2-AIT-2** suppressed resistance to all the penicillins tested with activities ranging from eight-fold reduction in MIC for ampicillin to four-fold reduction in MIC for methicillin, nafcillin and pipericillin. **2-AIT-2** also suppressed resistance to the carbapenem antibiotics imipenem and meropenem, reducing the MIC by eight-fold and four-fold respectively, and the cephamycin antibiotic cefoxitin, reducing the MIC by four-fold. Activity was also observed against the cephalosporins cefotaxime and ceftazidime, for which a four-fold and two-fold reduction in MIC were observed respectively. The only non ß-lactam antibiotic for which any reduction in MIC was observed was the glycopeptide vancomycin, for which a two-fold reduction in MIC was noted. **2-AIT-2** had no effect on the MIC of any other antibiotic tested including novobiocin, streptomycin, tobramycin, erythromycin, tetracycline and chloramphenicol.

2-AIT-2 was assessed for the ability to suppress resistance to oxacillin in a number of additional strains of MRSA. The strains examined included representative USA 100, 600 and 1000 MRSA clones (strains BAA-1753, BAA-1685 and BAA-1770 respectively) and a vancomycin intermediate (VISA) strain (strain 700789). **2-AIT-2** exhibited a lower MIC of 25 μM (11.9 μg/mL) against all the strains tested, with the exception of Xen 30, compared to the MIC observed against MRSA BAA-44. **2-AIT-2** was initially tested for oxacillin resistance

suppression activity at one quarter of its MIC (6.25 μM, 2.96 μg/mL) as per our original screening protocol. Only moderate activity was observed at this low concentration, so **2-AIT-2** was also tested at an increased concentration of 10 μM (4.7 μg/mL). As shown in Table 1, at this concentration, **2-AIT-2** reduced the MIC of all the strains tested by between eight-fold (for strains Xen-30, BAA-1770, and 33591) and two-fold. Growth curves were constructed to investigate the microbicidal activity of **2-AIT-2** at this concentration against all the strains tested and showed that the compound exhibited no microbicidal activity.

Table 1. Suppression of resistance to oxacillin for several strains of MRSA

Strain	Oxacillin MIC (μg/mL)	2AIT-2 MIC (μM)	Concentration tested (μM)	MIC (μg/mL)	Reduction in MIC
Xen 30	32	100	25	4	8-fold
BAA-1770	4	25	10	0.5	8-fold
33591	32	25	10	4	8-fold
BAA-811	16	25	10	4	4-fold
BAA-1753	16	25	10	8	2-fold
BAA-1685	32	25	10	16	2-fold
700787	32	25	10	16	2-fold

The effect of **2-AIT-2** on the potential of a methicillin susceptible strain of *S. aureus* (ATCC 29213) to evolve resistance to oxacillin was also investigated. It was first determined whether this *S. aureus* strain would evolve resistance to **2-AIT-2**. The MIC of **2-AIT-2** against this strain of *S. aureus* is 25 μM. Bacteria were cultured daily in 15, 20, 25 and 30 μM **2-AIT-2** and the highest concentration at which growth could be observed was used to inoculate the next passage of growth. The MIC was measured every 2-3 days. The highest concentration of **2-AIT-2** in which bacterial growth remained at 20 μM throughout a week of daily sub-culturing and the MIC determined by a standard broth microdilution method remained constant at 25 μM.

To investigate the evolution of resistance to oxacillin, *S. aureus* was cultured daily at 0.25, 0.5, 1 and 2 x the oxacillin MIC in the presence or absence of **2-AIT-2** (20 μM). Again the highest concentration at which growth could be observed was used to inoculate the next passage of growth and as the MIC evolved the concentrations of oxacillin were adjusted accordingly. The oxacillin MIC was measured every two-three days. The oxacillin MIC of the bacteria cultured in the presence of oxacillin alone increased from 0.125 μg/mL to 0.5 μg/mL after just two days and to 2 μg/mL over the 10-day period of the experiment. The addition of **2-AIT-2** (20 μM) to the culture slowed the evolution of the oxacillin MIC (**2-AIT-2** is added to the culture media for serial passage but not to the MIC assay). The MIC increased from 0.125 μg/mL to 0.25 μg/mL after two days and did not further increase for the remainder of the 10 days.

These results demonstrate that, during the time frame studied, this methicillin susceptible strain of *S. aureus* does not evolve resistance to **2-AIT-2**. Moreover, the presence of **2-AIT-2** considerably slows the evolution of resistance to oxacillin, from an increase in MIC of 16-fold for bacteria cultured in the absence of compound **3**, to an increase of just two-fold for those cultured in the presence of **2-AIT-2**.

The antibiotic suppression activity of **2-AIT-2** against a MDR resistant strain of *A. baumannii* (ATCC BAA-1605) has also been investigated. The MIC of **2-AIT-2** against this strain is 200 μM, and in the presence of 50 μM **2-AIT-2** the MIC of imipenem was reduced by four-fold (from 16 μg/mL to 4 μg/mL).

Activity of next generation analogues.

Library 1. Analogues **g–j** displayed biofilm dispersal activity against *A. baumannii* (ATCC 19606), exhibiting EC_{50} values of 45-60 μM (Table 1 Supporting Data) compared to 120 μM for **2-AIT-1.** Analogues **g, i** and **j** also inhibited biofilm formation by MRSA (ATCC BAA-44) via a non-microbicidal mechanism, exhibiting low micromolar IC_{50} values (4-10 μM). Members of this library displayed only modest penicillin G resistance suppression activity against MRSA (ATCC BAA-44) compared to **2-AIT-1** (two-fold reduction in MIC compared to eight-fold), though it is important to note that they were assayed at much lower concentrations than **2-AIT-1** due to their greater innate microbicidal activity.

Overall derivatization of the aromatic appendage of **2-AIT-1** increases biofilm dispersal activity against *A. baumannii* while also imparting greater antibacterial activity against both this bacterial strain, and against MRSA. Analogues possessing alkyl chain substituents proved to be the most active, and several analogues possess the ability inhibit MRSA biofilm formation at low micromolar concentrations. However, these compounds were found to be less effective at re-sensitizing MRSA to the effects of penicillin G, most probably

due to the fact that their increased toxicity meant that they had to be assayed at much lower concentrations to ensure the reduction in MIC observed is attributed to re-sensitization activity alone.

Library 2. The most potent compound identified from this library for inhibition of MRSA (ATCC BAA-44) biofilm formation through a non-microbicidal mechanism was compound **e**, which exhibited an IC_{50} value of 1.42 μM (Table 2 Supporting Data). A second generation of this library consisting of compounds with substitution about the phenyl ring at the 4-position of the 2-aminoimidazole led to the identification of an analogue (**t**), with increased activity (IC_{50} 1.34 μM). Analogues **c**, **e**, and **v** exhibited biofilm inhibitory activity against *A. baumannii* (ATCC 19606) (IC_{50} values 15.57, 11.28 and 20.35 μM respectively). These compounds also possess increased ability to disperse pre-formed *A. baumannii* biofilms compared to **2-AIT-1**, with EC_{50} values of 50.03, 44.61 and 60.80 μM respectively.

Compounds **c**, **l** and **m** re-sensitized MRSA to the effects of oxacillin by four-fold (compared to two-fold for **2-AIT-1**). The degree of synergy between the lead compounds and oxacillin was determined using the checkerboard assay, which allows the fractional inhibitory concentration (FIC) to be calculated. The sum of the FICs was calculated ($\Sigma FIC = FIC_{Cmpd} + FIC_{Oxa}$ where FIC_{Cmpd} represents "MIC of compound in the combination/MIC of compound alone" and FIC_{Oxa} represents "MIC of oxacillin in the combination/MIC of oxacillin alone") and determined to be 0.25, 0.19 and 0.5 for compounds **c**, **l** and **m** respectively, confirming that these three compounds work synergistically with oxacillin

Library 3. Initial screening for the ability to inhibit the formation of biofilms by *A. baumannii* and *E. coli* identified several compounds that affected *E. coli* biofilm formation but did not affect biofilm formation by *A. baumannii*. IC_{50} values for the lead compound, compound **b** against *E. coli* was determined to be 36.9 μM. Compound **b** was also able to disperse pre-established *E. coli* biofilms, exhibiting an EC_{50} of 120 μM.

Library 4. Compound **h** from this library was the most potent inhibitor of MRSA (ATCC BAA-44) biofilm formation that was shown to be acting via a non-microbicidal mechanism, exhibiting an IC_{50} value of 3.71 μM (Table 3 Supporting Data). Several other compounds displayed lower IC_{50} values (the most active being compound m, IC_{50} = 1.99 μM) but were shown to possess some microbicidal activity at the IC_{50} concentration. Additionally several compounds exhibited the ability to inhibit biofilm formation by MDR *A. baumannii* (ATCC BAA-1605) through a non-microbicidal mechanism, with compound **e** displaying an IC_{50} of 25.85 μM. A number of compounds form this library suppressed resistance of MRSA (BAA-44) to oxacillin by up to four-fold, though none were as active as the parent compound **2-AIT-2**.

Library 5. The introduction of an amide or sulfonamide group at the 2-position of **2-AIT-2** resulted in a complete loss of biofilm inhibitory activity against MRSA (ATCC BAA-44) however analogues with alkyl substituents at the 2-position, retained biofilm inhibitory activity against this MRSA strain (Table 4 Supporting Data), with compound **c**, in which the substituent is an octyl group, having comparable activity to **2-AIT-2** (IC_{50} 4.3 μM). Several members of this library displayed biofilm inhibitory activity against a number of other MRSA strains investigated (Table 5 Supporting Data), the most active compounds identified were: **e**, **g**, **l**, **m** and **n**, all exhibiting low micromolar IC_{50} values, though it is yet to be determined if all are acting through a non-microbicidal mechanism.

Several library 5 analogues were also able to inhibit biofilm formation by two strains of *A. baumannii* (antibiotic sensitive strain ATCC 19606 and antibiotic resistant strain ATCC BAA-1605) (Table 6 Supporting Data). As found for MRSA, introduction of an amide or sulfonamide group at the 2-position resulted in a complete loss of activity. Analogues with alkyl substituents at this position were able to inhibit biofilm formation by these strains, with compound **b**, which possesses a hexyl substituent, being the most active member of this library, exhibiting IC_{50} values of 32.3 μM and 25.7 μM for strains 19606 and BAA-1605 respectively.

As for library 4, a number of compounds form this library suppressed resistance of MRSA (BAA-44) to oxacillin by up to four-fold at concentrations at which they did not display microbicidal activity, though again none were as active as the parent compound **2-AIT-2**.

Library 6. All library 6 analogues tested to date exhibited low micromolar IC_{50} values for inhibition of MRSA (ATCC BAA-44) biofilm formation (Table 7 Supporting Data), with the most potent being compound **a**, which has an IC_{50} value of 1.44 μM.

Library 8 Initial testing of this library revealed that these analogues possess higher microbicidal activities than do mono- or di- substituted 2-aminoimidazole derivatives. We therefore screened the library for antibiotic activity against MRSA, methicillin sensitive *S. aureus*, *A. baumannii* and *Escherichia coli*, and discovered that this class of small molecules was microbicidal primarily against Gram-positive strains. Compounds **d**, **n** and **o** were the most active, with MIC values (μg/mL) of 2, 4, 64, >256; 32, 32, 64, 64 and 4, 8, 16, >256 against MRSA,

MSSA, *A. baumannii* and *E. coli*, respectively (Table 8 Supporting Data). Due to the higher activity against Gram-positive strains, we next investigated lead compounds from this library for antimicrobial activity against a number MRSA strains isolated from a nosocomial environment. The lead compounds displayed MIC values as low as 2 μg/mL (Table 9 Supporting Data).

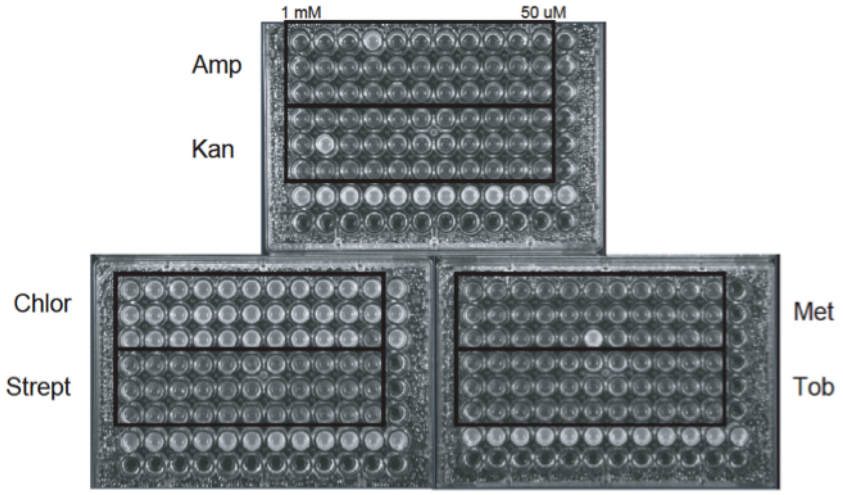


Figure 11: *C. elegans* fecundity assay using **2-AIT-1** at concentrations ranging from 1 mM to 50 µM. Six different antibiotics were used at selective concentrations: ampicillin (Amp), kanamycin (Kan), chloramphenicol (Chlor), streptomycin (Strept), methicillin (Met), and tobramycin (Tob). Chloramphenicol was used as a negative control and remained opaque throughout the assay.

Aim 3. Evaluate active 2-AIT derivatives for in vitro toxicity. Models will include epidermal cell toxicity, model organism toxicity (*Caenorhabditis elegans*), and hemolysis.

The toxic effects of **2-AIT-1** have been evaluated using a methylthiazolyldiphenyl-tetrazolium (MTT) assay to measure the cytotoxic effects of epidermal exposure, and a *Caenorhabditis elegans* fecundity assay to observe the reproductive effects of environmental exposure.[7]

The MTT assay measures cellular proliferation using the HaCaT keratinocyte cell line, to determine if **2-AIT-1** exhibited any cytotoxic effects through epidermal exposure. HaCaT cells were externally exposed to concentrations of **2-AIT-1** from 2.5 mM to 1.2 µM. The concentration of 2-AIT-1 at which 50% toxicity ($TCID_{50}$) and no toxicity (endpoint) as compared to the controls was recorded. **2-AIT-1** exhibited a $TCID_{50}$ of 119 µM with an endpoint of 36 µM.

The *C. elegans* fecundity assay is a qualitative assay used to determine an estimated concentration threshold (ECT) for each compound. The ECT is defined as the concentration at which the media (*Escherichia coli* HB101/S medium) in the wells is no longer clear enough to see the worms, even under a microscope. Media clearing indicates that the worms are able to feed and reproduce across more than one generation without experiencing any detrimental effects from their exposure to the compound at the tested concentration. Beyond the ECT for a compound, the wells remain opaque due to either death of the worms or a toxic effect that has damaged their ability to reproduce or develop.

Preliminary testing of **2AIT-1** was carried out at concentrations at which it exhibits anti-biofilm and antibiotic resistance suppression activity, from 100 µM to 1 µM. After six days of incubation, **2-AIT-1** was found to have no toxicity at every tested concentration indicated by complete clearing of the media for each replicate and controls confirmed that the clearing was the result of the feeding activity of the large number of *C. elegans* present in the well across various developmental stages. Follow up assays over an expanded concentration range revealed that **2-AIT-1** has an apparent ECT of 750 - 800 µM.

2AIT-1 was also investigated for changes in toxicity under adjuvant conditions with several antibiotics (ampicillin, kanamycin, streptomycin, methicillin, and tobramycin). A set of control assays confirmed that the antibiotic concentrations being used did not have a toxic effect on the worms when applied alone.

After confirming that the antibiotics would not bias the results, a fecundity assay was conducted with the addition the antibiotics at full working concentrations and **2-AIT-1** concentration steps of 100 µM and one 50 µM step. As the chloramphenicol solution had deleterious reproductive effects on the worms in a control assay, it was included as a control. After a week of incubation, all of the antibiotic and adjuvant combinations showed remarkable clearing (Figure 11), confirming that the combination of **2-AIT-1** with various antibiotics is non-toxic to *C. elegans* at concentrations above that at which it exerts antibiotic resistance suppression activity.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed synthetic routes to access various substitution patterns about the 2-aminoimidazole ring.
- Identified several novel non-microbicidal compounds that inhibit biofilm formation by MRSA and MDR *A. baumannii* with low micromolar IC_{50} values.
- Identified a number of novel compounds that disperse *A. baumannii* biofilms.
- Characterized the antibiotic resistance suppression activity of a new lead compound.
- Demonstrated using cellular and model organism toxicity assays that the lead compound 2-AIT-1 does not exhibit eukaryotic toxicity, both alone and in combination with antibiotics, at concentrations well above those at which it exhibits anti-biofilm and antibiotic resistance suppression activity.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Manuscripts:

- Su, Z., Peng, L., Worthington, R.J., and Melander, C. Evaluation of 4,5-Disubstituted-2-Aminoimidazoles for Dual Antibiofilm/Antibiotic Resensitization Activity. *ChemMedChem*, 2011, **6**, 2243-225.
- Su, Z., Peng, L., and Melander, C. A Modular Approach to the Synthesis of 1,4,5-Substituted-2-Aminoimidazoles. *Tetrahedron Letters*, 2012, 53 (10), 1204-1206.
- Stowe, S.D., Tucker, A.T, Rogers, S.A., Richards, J.J., Melander, C., and Cavanagh, J. Evaluation of 2-Aminoimidizaole Toxicity in both Cellular and Model Organism Systems. *Drug and Chemical Toxicology*, January 31, 2012. (doi:10.3109/01480545.2011.614620)

Presentations:

Poster: Development of Molecules that Disarm Multiple Antibiotic Resistance Mechanisms, Roberta J. Worthington and Christian Melander, ICAAC, Chicago, Sep 17-20, 2011.

Poster: Suppression of Antibiotic Resistance by Modulating Response Regulator Function, Roberta J. Worthington and Christian Melander, GRC New Antibacterial Discover and Development, Lucca Italy, April 15-20, 2012.

Abstracts:

ICAAC, San Francisco, Sep 9-12, 2012, submitted

CONCLUSION:

New approaches are required to control multi-drug resistant (MDR) bacterial infections in military medical facilities, as injured Warfighters are highly susceptible to such infections.

We are attempting to address this problem through the development of a 2-aminoimidazole compound that, at sub-micromolar levels and in conjunction with conventional antibiotics, will inhibit and disperse biofilms from both Gram-positive and Gram-negative pathogens, and re-sensitize MDR variants of these pathogens to FDA approved antibiotics.

During the first year of this award we have developed synthetic routes to access various substitution patterns about a 2-aminoimidazole ring and constructed a number of focused chemical libraries. Many of these compounds have been evaluated for antibiotic resistance suppression and anti-biofilm activity with a number of analogues exhibiting increased activity compared to the initial lead compound. Completion of in vitro screening of all compounds, including against clinical isolates obtained from Walter Reed Army Institute of Research (WRAIR) will be completed shortly.

We have also demonstrated using cellular and model organism toxicity assays that the lead compound 2-AIT-1 does not exhibit eukaryotic toxicity, both alone and in combination with antibiotics, at concentrations well above those at which it exhibits anti-biofilm and antibiotic resistance suppression activity. This now allows us to take the lead compounds identified from the in vitro screening assays into in vivo experiments in the second year of this award, using animal models of infection (mouse puncture and pig partial thickness and rat osteomyelitis) developed at WRAIR.

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6. Su, Z., Peng, L., and Melander, C. A Modular Approach to the Synthesis of 1,4,5-Substituted-2-Aminoimidazoles. Tetrahedron Letters, 2012, 53 (10), 1204-1206.
7. Stowe, S.D., Tucker, A.T, Rogers, S.A., Richards, J.J., Melander, C., and Cavanagh, J. Evaluation of 2-Aminomidizaole Toxicity in both Cellular and Model Organism Systems. Drug and Chemical Toxicology, January 31, 2012. (doi:10.3109/01480545.2011.614620)

APPENDICES:

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

Table 1. Antibiotic and anti-biofilm activity of lead library 1 analogues.

	A. baumannii MIC		*A. baumannii* EC_{50} (µM)	MRSA IC_{50} (µM)
Compound	**µM**	**µg/mL**		
g	25	10.92	59.61 ± 7.28	9.86 ± 2.85
h	6.25	2.77	53.90 ± 3.39	[a]
i	12.5	5.74	44.70 ± 2.25	8.55 ± 1.06
j	12.5	6.95	49.38 ± 4.83	4.50 ± 0.53
n	25	11.78	[b]	[b]

[a] Microbicidal [b] Not active

Table 2. Biofilm inhibition data for library 2 against MRSA (ATCC BAA-44).

Compound	IC_{50} (µM)	Compound	IC_{50} (µM)	Compound	IC_{50} (µM)	Compound	IC_{50} (µM)
a	25.73±0.28	**g**	1.89±0.05	**m**	8.20±0.10	**s**	1.52±0.30
b	2.25±0.04	**h**	2.95±0.37	**n**	1.97±0.03	**t**	1.34±0.10
c	1.91±0.02	**i**	2.19±0.03	**o**	1.50±0.21	**u**	1.96±0.06
d	2.01±0.01	**j**	10.08±0.13	**p**	1.45±0.05	**v**	1.95±0.05
e	1.42±0.18	**k**	2.23±0.07	**q**	1.89±0.11		
f	2.10±0.13	**l**	2.15±0.23	**r**	1.39±0.08		

Table 3. Anti-biofilm and antibiotic resistance suppression activity of library 4.

	MDRAB (ATCC BAA-1605)	MRSA (ATCC BAA-44)					
Compound	**IC_{50} (µM)**	**IC_{50} (µM)**	**MIC (µg/mL)**	**MIC (µM)**	**Resensitization with Oxacillin (fold)**	**Resensitization with Penicillin G (fold)**	**ΣFIC**
a		4.10±0.30	4	7.27	2	0	0.625
b		2.11±1.03	2	3.38	0	0	0.625
c		2.95±0.67	2	3.38	2	0	0.5
d		3.75±2.30	2	3.3	2	0	0.5
e	25.85±3.45[a]	2.82±0.54	8	12.63	4	2	0.5
f		3.46±0.84	4	6.76	2	0	0.625
g		3.57±0.84	4	6.89	0	0	1
h		3.71±0.84[a]	8	13.46	2	0	0.531
i		8.70±1.60	16	27.86	2	0	0.562
j	59.19±20.36	4.39±0.62	4	6.82	0	0	0.375
k		3.94±0.33	4	7.04	0	0	1
l		3.58±0.98	2	3.43	0	0	0.625
m		1.99±0.21	4	6.39	4	2	0.375
n		2.31±0.60	4	6.66	2	2	0.375
o		13.94±2.67	16	29.67	2	0	0.562
p		7.22±1.84[a]	16	29.67	4	2	0.375
q	69.38±6.82[a]	6.03±1.99	8	13.58	2	2	0.3125
r		10.88±1.90	16	28.82	2	0	0.75
s		5.74±0.53	8	14.41	0	0	0.531
t	50.45±13.38[a]	4.85±0.71	16	26.43	2	2	0.375
u		2.61±0.91	8	13.22	2	2	0.5
v		19.18±1.78	32	58.15	0	0	0.75
w		4.22±0.98[a]	8	13.33	0	0	0.5

[a] Non- microbicidal

Table 4. Activity of library 5 analogues against MRSA (ATCC BAA-44)

Compound	MIC (μg/mL)	∑FIC with oxacillin	Biofilm inhibition IC_{50} (μM)
a	16	> 0.5	23.6 ± 4.9
b	4	> 0.5	6.9 ± 1.1
c	4	> 0.5	4.3 ±0.9
d	16	> 0.5	8.9 ±1.7
f	>128	> 0.5	No Activity
g	>128	> 0.5	No Activity

Table 5. Biofilm inhibition activity of library 5 analogues against *A. baumannii.*

Compound	Biofilm inhibition IC_{50} (μM)	
	ATCC 19606	**ATCC BAA-1605**
2-AIT-2-5a	58.6 ± 4.8	Toxic
2-AIT-2-5b	32.2 ± 9.6	25.7 ± 0.13
2-AIT-2-5c	47.7 ± 11.9	40.7 ± 4.2
2-AIT-2-5d	45.5 ± 9.7	55.5 ± 0.8
2-AIT-2-5f	No Activity	No Activity
2-AIT-2-5g	No Activity	No Activity

Table 6. Biofilm inhibition activity of library 5 analogues against additional MRSA strains.

Compound	43300	BAA-1685	BAA-1770
2-AIT-2	33.62 ± 2.82	21.61 ± 1.27	Toxic
a	42.16 ± 3.98	20.01 ± 1.62	36.33 ± 6.35
b	NA	NA	NA
c	61.04 ± 9.49	47.60 ± 13.83	52.65 ± 9.17
d	36.09 ± 2.33	24.56 ± 2.01	30.15 ± 5.71
e	10.70 ± 1.29	5.73 ± 3.66	10.41 ± 1.43
f	11.35 ± 0.83	9.21 ± 1.05	8.64 ± 2.07
g	7.35 ± 1.36	3.68 ± 0.92	5.32 ± 1.47
h	Toxic	Toxic	76.42 ± 3.98
i	NA	NA	NA
j	Not tested	Not tested	Not tested
k	14.63 ± 0.54	8.13 ± 1.08	11.74 ± 1.88
l	5.90 ± 0.66	5.48 ± 0.77	4.40 ± 0.76
m	8.94 ± 1.11	4.50 ± 1.11	7.35 ± 0.72
n	8.33 ± 1.04	7.53 ± 0.06	8.33 ± 3.33
o	NA	NA	NA
p	Not tested	Toxic	66.80 ± 7.75

NA – Not active. Toxic – these compounds have exhibited steep dose response curves such that IC_{50} values could not be accurately determined.

Table 7. Antibiotic and anti-biofilm activity of library 6 analogues.

Compound	MRSA BAA-44 IC_{50} (μM)	MRSA BAA-44 MIC (μM)	MDRAB BAA-1605 MIC (μM)
a	1.14	50	50
b	2.25	6.25	100
c		3.125	100
d	1.85	6.25	100
e	2.14	6.25	50
f	2.35	6.25	25
j		3.125	200
k	2.89	12.5	25

Table 8. Antibiotic activity of library 8.

Compound	*A. baumannii*	*E. coli*	MSSA	MRSA	Compound	*A. baumannii*	*E. coli*	MSSA	MRSA
a	128[a]	256	256	256	**i**	>256	>256	>256	>256
b	>256	>256	16	8	**j**	>256	>256	4	4
c	>256	>256	>256	>256	**k**	>256	>256	>256	>256
d	64	>256	4	2	**l**	>256	>256	8	4
e	>256	>256	>256	128	**m**	>256	>256	>256	>256
f	>256	>256	>256	>256	**n**	64	64	32	32
g	>256	>256	32	4	**o**	16	>256	8	4
h	>256	>256	>256	>256					

[a]MIC values in $\mu g/mL$.

Table 9. Antibiotic activity of lead compounds from library 8 against different MRSA strains.

Strain	**Compound d**	**Compound o**	**Strain**	**Compound d**	**Compound o**
BAA 1770	8	8	**BAA 44**	2	4
BAA 1556	4	8	**33591**	8	8
BAA 811	2	8	**700789**	4	8
BAA 1685	4	8	**43300**	2	8
BAA 1753	4	8			

[a]MIC values in $\mu g/mL$.